



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In the Application of:	)	
	)	
Lawton <i>et al.</i>	)	
	)	Art Unit: 1645
Serial No.: 10/054,647	)	
	)	Examiner: Ford, V.
Filed: January 22, 2002	)	
	)	Conf. No. 9151
For: Compositions and Methods for Detection	)	
of <i>Ehrlichia canis</i> and <i>Ehrlichia</i>	)	Atty. Docket No.: 00-1278-C
<i>chaffeensis</i> Antibodies	)	

REPLY BRIEF

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<b><i>chaffeensis</i> Antibodies</b>	)	

**REPLY BRIEF**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

This reply brief is filed in response to the Examiner's answer mailed on January 28, 2005. It is believed that no fee is due in connection with this filing. However, if a fee is due, the Commissioner is authorized to charge our Deposit Account No. 13-2490.

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### **REAL PARTY IN INTEREST**

The real party in interest is IDEXX Laboratories, Inc., Westbrook, Maine, to whom this application is assigned.

### **RELATED APPEALS AND INTERFERENCES**

A related appeal brief has been filed on July 23, 2004, in U.S. Serial No. 09/765,739, of which this application is a divisional. An appeal brief has also been filed on October 21, 2004, in U.S. Serial No. 10/054,354, which is also a divisional application of U.S. Serial No. 09/765,739. No decisions by a court or the Board have been issued in these related applications. Applicant is aware of no other related appeals, interferences, or judicial proceedings concerning this application.

### **STATUS OF CLAIMS**

Claims 1-13 are pending and stand rejected. Claims 1-13 are being appealed. A copy of the claims is attached in Appendix A.

### **STATUS OF AMENDMENTS**

No amendments were presented after final.

### **SUMMARY OF THE INVENTION**

A summary of the invention can be found in the Appeal Brief.

### **GROUND OF REJECTION TO BE REVIEWED ON APPEAL**

- I. Claims 1-13 stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking written description.
- II. Claims 1-13 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement.

- III. Claims 1-13 stand rejected under 35 U.S.C. §102(a) as allegedly anticipated by Rikihisa *et al.* WO 99/13720 (“Rikihisa”).
- IV. Claims 1-9 stand rejected under 35 U.S.C. §112, second paragraph as allegedly indefinite.

## ARGUMENT

### I. **Claims 1-13 have adequate written description under 35 U.S.C. §112, first paragraph.**

#### **Claims 1-13**

Claims 1-13 stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking written description. Applicants respectfully traverse the rejection.

The examiner's answer states that the Office is interpreting the claimed compositions of matter and articles of manufacture to comprise an isolated polypeptide that binds an anti-*Ehrlichia* antibody. The Office therefore concludes that the claims read on isolated, whole *Ehrlichia* proteins that are not limited to the amino acid sequence of SEQ ID NO:2 and the claimed amino acid substitution variants. The examiner's answer concludes that the claimed phenotypically silent amino acid substitution variants and conservative amino acid substitution polypeptide variants do not have adequate written description because the claims encompass substitution variants of whole *Ehrlichia* proteins, which are not described by the specification.

Claims 1-9 recite compositions comprising an isolated polypeptide *consisting essentially* of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant or conservative amino acid substitution variant thereof that specifically bind to an anti-*Ehrlichia* antibody.

The transitional phrase "consisting essentially of" means that the elements or element modified by the phrase covers the expressly recited subject matter and unrecited subject matter that does not materially affect the basic and novel properties of the invention. *See PPG Indus. v. Guardian Indus. Corp.*, 156 F.3d 1351, 1354; 48 U.S.P.Q.

2d 1351, 1351 (Fed. Cir. 1998). “Consisting essentially of” occupies a middle ground between closed claims using a “consisting” transitional phrase and open claims having a “comprising” transitional phrase. *See id* at 1354, 1354.

The examiner’s answer asserts that the claims read on whole *Ehrlichia* proteins, which are about 275 to about 310 amino acids in length. It is appellants’ position that the inclusion of all of the amino acids of the whole *Ehrlichia* proteins would affect the basic and novel characteristics of the claimed 20 amino acid fragments of whole *Ehrlichia* polypeptides. Where the inclusion of unrecited ingredients affects the basic and novel characteristics of the claimed composition, these unrecited ingredients are not to be considered to be within the scope of the claim. *See e.g., PPG Indus.* at 1356, 1356. Therefore, the claims should not be interpreted to encompass whole *Ehrlichia* proteins.

The Office has alleged that all of the basic and novel characteristics of the claimed composition do not appear in the claims and therefore, has dismissed the appellants’ assertion of the basic and novel characteristics of the claimed compositions. *See e.g., Examiner’s answer*, page 18, line 18 to page 19, line 3. However, there is no requirement that the basic and novel characteristics appear in the claims using a “consisting essentially of” transitional phrase. *See e.g., AK Steel Corp. v. Sollac*, 344 F3d 1234, 1239; 68 U.S.P.Q.2d 1280, 1283 (Fed. Cir. 2003) (stating “[t]o determine [the basic and novel] properties, we need look no further than the patent specification.”). Therefore, the Office must consider the claims to recite polypeptides consisting essentially of SEQ ID NO:2, and not polypeptides comprising SEQ ID NO:2.

The basic and novel characteristics of the claimed polypeptides are that they bind an anti-*Ehrlichia* antibody and that they provide greater sensitivity and greater specificity

in assays than whole *Ehrlichia* proteins. The novelty of the polypeptides is not just the fact that they bind anti-*Ehrlichia* antibodies, but that they do so with greater sensitivity and specificity than whole *Ehrlichia* proteins.

The specification teaches that:

Indirect immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays (ELISA) are frequently used as aids in the diagnosis of these diseases. These assays measure or otherwise detect the binding of anti-*Ehrlichia* antibodies from a patient's blood, plasma, or serum to infected cells, cell lysates, or purified *Ehrlichia* proteins. However, currently known assays for detecting anti-*Ehrlichia* antibodies or fragments thereof are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. See page 2, line 21 through page 3, line 1 (emphasis added).

Additionally, in Example 1, the specification provides a working example that compares the sensitivity and specificity of polypeptides of SEQ ID NO:1 and SEQ ID NO:2 to the sensitivity and specificity of whole, partially purified *Ehrlichia* proteins. In the testing of 70 samples, the polypeptides of SEQ ID NO:1 and SEQ ID NO:2 exhibited a sensitivity of 98.5% and a specificity of 100% in contrast to the whole, partially purified *Ehrlichia* proteins, which exhibited a sensitivity of 75.3% and a specificity of only 60%. See specification, paragraph spanning page 20 and 21.

Furthermore, the declaration of Dr. Chandrashekar confirms that the claimed polypeptides are more sensitive and specific than whole *Ehrlichia* proteins. See paragraphs 2-3 and 6-7 (of record; copy attached in Appendix B).

The addition of amino acids to the claimed isolated polypeptides so they encompass whole *Ehrlichia* proteins would materially affect the basic and novel characteristics of the polypeptides. That is, use of whole *Ehrlichia* proteins would result in assays that are less sensitive and less specific than those disclosed in the instant

specification. As such, the claims cannot be read so that the claimed isolated polypeptides encompass whole *Ehrlichia* proteins.

Finally, the Office Action asserts that the general knowledge in the art concerning species does not provide any indication of how the structure of a limited number of other species is representative of unknown species. However, the general knowledge in the art recognizes that sequences from several species can be aligned and examined for positions along the sequence that are retained from species to species, *i.e.*, that are “conserved.”

The partial structure of claimed variants are known, *i.e.*, sequences that having at least 85% identity to SEQ ID NO:2. See specification, page 5, lines 8-11. Therefore, variants have about 17 or more amino acids in common with the 20 amino acid long SEQ ID NO:2. Additionally, appellants have provided 7 sequences (SEQ ID NOs:1-7) that can be aligned by one of skill in the art to form a consensus sequence. A consensus sequence is the compilation of multiple related sequence alignments. The consensus sequence shows which amino acids positions are conserved, and which amino acids positions are variable. When the sequences are aligned highly conserved amino acids and partially conserved amino acids are revealed. See Table 1.

Table 1.

SEQ ID NO	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	K	S	T	V	G	V	F	G	L	K	H	D	W	D	G	S	P	I	L	K
2	N	T	T	T	G	V	F	G	L	K	Q	D	W	D	G	A	T	I	K	D
3	N	T	T	V	G	V	F	G	L	K	Q	N	W	D	G	S	A	I	S	N
4	N	P	T	V	A	L	Y	G	L	K	Q	D	W	N	G	V	S	A		
5	N	T	T	V	G	V	F	G	L	E	Q	D	W	D	R	C	V	I	S	
6	N	P	T	V	A	L	Y	G	L	K	Q	D	W	E	G	I	S	S		
7	N	T	T	T	G	V	F	G	L	K	Q	D	W	D	G	S	T	I	S	

Table 1 demonstrates that positions 3, 8, and 13 of SEQ ID NO:2 are highly conserved across the seven sequences (dark gray columns). Additionally, positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15 are partially conserved across the 7 sequences (light gray columns). That is, only 2 different amino acids appear in these positions. For instance, only K or N appear as amino acids in position 1 across the seven sequences. One of skill in the art would recognize that variants should likely retain the amino acids at positions 3, 8, and 13 and that one of two amino acids should likely be present at positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15. One of skill in the art would also recognize that amino acids at position 2, 14, and 16-20 could tolerate a greater range of amino acid substitutions. Therefore, the specification combined with the general knowledge in the art provides an indication of how the structures of the disclosed species are representative of the claimed species.

Finally, the examiner's answer asserts that undue experimentation would be required to select amino acid substitution variants that retain the recited function due to the variable number of modification that can be made within the sequence of SEQ ID NO:2. Appellants have addressed the Office's allegations regarding undue experimentation below, in the response to the enablement rejection of claims 1-13. These arguments are encompassed herein.

Claims 1-13 have adequate written description in the specification. The claims are properly interpreted to encompass compositions comprising an isolated polypeptide *consisting essentially* of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant or conservative amino acid substitution variant thereof that specifically bind to an

anti-*Ehrlichia* antibody. Additionally, the species disclosed in the specification are representative of the claimed species.

### **Claims 10-13**

Claims 10-13 specifically state that the claimed polypeptides are 20 amino acids in length. As such, the claims certainly do not read on whole *Ehrlichia* proteins. Claims 10-13 encompass a 20 amino acid long polypeptide comprising SEQ ID NO:1 and phenotypically silent or conservative amino acid substitution variants thereof. As described above for claims 1-13, the specification provides an adequate written description of the claimed amino acid substitution variants of SEQ ID NO:1 and those arguments are repeated herein in their entirety.

Appellants respectfully request withdrawal of the rejection.

### **II. Claims 1-13 are enabled under 35 U.S.C. §112, first paragraph.**

Claims 1-13 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement. Applicants respectfully traverse the rejection.

### **Claims 1-13**

Initially, the examiner's answer states that the Office is interpreting the claimed compositions of matter and articles of manufacture to encompass whole *Ehrlichia* proteins. Appellants, however, have provided detailed reasoning demonstrating that the claims do not read on whole *Ehrlichia* proteins because, *inter alia*, the addition of amino acids to the claimed isolated polypeptides so they encompass whole *Ehrlichia* proteins would materially affect the basic and novel characteristics of the polypeptides (see response to written description rejection, above). Appellants repeat these same arguments in their entirety in regards to this enablement rejection.

The examiner's answer asserts that variants of SEQ ID NO:2 are not enabled and states that "[t]here is no guidance provided as to which amino acids can be added, deleted or substituted and the polypeptide would retain its biological function." The examiner's answer also states that "predictability of which [amino acid] changes can be tolerated in a polypeptide's amino acid sequence and still retain similar activity requires a knowledge with regard to which amino acids in the polypeptide's sequence, if any, are tolerant of modification and which are conserved."

As discussed in the response to the written description rejection, the specification does indeed provide information as to which amino acids substitutions can be tolerated in the amino acid sequence of SEQ ID NO:2 so that the polypeptide retains specific binding to an *Ehrlichia* antibody. Table 1 demonstrates which amino acids in SEQ ID NO:2 are tolerant to modifications which are conserved.

The Office asserts that undue experimentation would be necessary to practice the invention. *In re Angstadt* examined the issue of undue experimentation in unpredictable arts. 537 F.2d 498, 502; 190 U.S.P.Q. 214, 218 (C.C.P.A. 1976). In *Angstadt*, the claims were directed to the preparation of a reaction mixture comprising hydroperoxides using a complex catalyst comprising a transition metal salt. The court examined whether a reasonable number of transition metal salts were disclosed in the specification to enable the claims. The first issue was "whether, in an unpredictable art, section 112 requires disclosure of a test with *every* species covered by a claim." *Id.* (emphasis in the original). The court found that "appellants are *not* required to disclose *every* species encompassed by their claims even in an unpredictable art." *Id.* (emphasis in the original). This is because (1) such a requirement would necessitate a patent application with thousands of

examples or the disclosure of thousands of catalysts; and (2) more importantly, such a requirement would force an inventor seeking adequate patent protection to carry out a prohibitive number of actual experiments. This could result in discouraging inventors from filing patent applications in unpredictable fields and allowing infringers to readily avoid literal infringement by merely finding an analogous catalyst. *See id.*

The court found that the appellant provided those skilled in the art with a *large but finite list* of transition metal salts from which to choose in preparing a complex catalyst. The court stated that:

The process discovered by appellants is not complicated, and there is no indication that special equipment or unusual reaction conditions must be provided when practicing the invention. One skilled in this art would merely have to substitute the correct mass of a transition metal salt for the transition metal salts disclosed in appellants' 40 runs. Thus, we have no basis for concluding that persons skilled in this art, armed with the specification and its 40 working examples, would not easily be able to determine which catalyst complexes within the scope of the claims work to produce hydroperoxides and which do not. *Id.* at 503, 218.

While appellants in the instant application have not provided 40 working examples, they have provided working examples and have provided a total of 7 related species that provide guidance to one of skill in the art as to where phenotypically silent and conservative amino acid substitutions can be made along the length of the claimed polypeptides. Similar to the situation in *Angstad*, a finite explanation of the amino acid substitutions allowed, and a teaching of how to make and how to use the claimed polypeptides has been provided. The process of making and testing 20 amino acid long polypeptides with up to three amino acid substitutions is not complicated and no special equipment or unusual reaction conditions are required. One skilled in the art would merely have to substitute one to three amino acids in SEQ ID NO:2. Guidance as to

which amino acids could be substituted is provided by the alignment of the disclosed species. *See e.g.*, Table 1. The experimentation required to determine which substitutions will produce functional polypeptides would not be undue and certainly would not "require ingenuity beyond that to be expected of one of ordinary skill in the art." *Fields v. Conover*, 443 F.2d 1386, 1390-91, 170 U.S.P.Q.276, 279 (C.C.P.A.1971).

The court in *Angstad* states that the applicant does not have to provide guidance which will enable one skilled in the art to determine, *with reasonable certainty before performing the reaction*, whether the claimed product will be obtained, because then *all* "experimentation" is "undue," since the term "experimentation" implies that the success of the particular activity is *uncertain*. *See id.* at 503, 219. The court asserts that such a reading of the requirements under section 112 would be contrary to the basic policy of the Patent Act, which is to encourage disclosure of inventions and thereby to promote progress in the useful arts. To require disclosures in patent applications to transcend the level of knowledge of those skilled in the art would stifle the disclosure of inventions in technological fields that are understood imperfectly. *See id.*

Appellants have broadly disclosed a class of polypeptides. But for this disclosure the public may have been deprived of the knowledge of appellants' compositions. In this art the disclosure of several sequences that provide a consensus sequence is "reasonable," even if the end result is uncertain. Those of skill in the art would know how to make up to 3 amino acid substitutions that are conservative or phenotypically silent within the scope of the claims, within the ambit of the types and amount of experimentation which the uncertainty of this art makes inevitable.

The Office asserts that no declaration or other evidence has been made of record establishing the amount of experimentation necessary to make and use the claimed polypeptides. The making of 20 amino acid long polypeptides and making substitutions of up to three amino acids in such polypeptides is well known to those of skill in the art. *See e.g.,* Bowie (of record). Finally, the Office states that all amino acids can be substituted along the length of SEQ ID NO:2 resulting in a great number of variants. General knowledge in the art combined with the teachings of the specification would point one of skill in the art to functional substitutions. For example, Table 1 shows that position 1 of SEQ ID NOs:1-7 is K or N. One of skill in the art would know that while position 1 could be substituted with amino acids other than K and N, it would be advantageous to retain a K or N at position 1 to retain specific binding to an anti-*Ehrlichia* antibody.

### **Claims 10-13**

Claims 10-13 specifically state that the claimed polypeptides are 20 amino acids in length. As such, the claims certainly do not read on whole *Ehrlichia* proteins. Claims 10-13 encompass a 20 amino acid long polypeptide comprising SEQ ID NO:1 and phenotypically silent or conservative amino acid substitution variants thereof. As described above for claims 1-13, the claimed amino acid substitution variants of SEQ ID No:1 are enabled and those arguments are repeated herein in their entirety.

Therefore, claims 1-13 are indeed enabled and appellants respectfully request withdrawal of the rejection.

### **III. Claims 1-13 are novel over Rikihisa under 35 U.S.C. §102(a)**

Claims 1-13 stand rejected under 35 U.S.C. §102(a) as allegedly anticipated by Rikihisa *et al.* Applicants respectfully traverse the rejection.

### **Claims 1-13**

The examiner's answer asserts that the claims read on whole *Ehrlichia* proteins due to the use of the transitional phrase "consisting essentially of" in relation to polypeptides of SEQ ID NO:2. Appellants, however, have provided detailed reasoning above that the claims do not read on whole *Ehrlichia* proteins because, *inter alia*, the addition of amino acids to the claimed isolated polypeptides so they encompass whole *Ehrlichia* proteins would materially affect the basic and novel characteristics of the polypeptides (see response to written description rejection, above). Appellants repeat these same arguments in their entirety in regards to this novelty rejection. Appellants assert that the claims encompass only specific, isolated 20 amino acid long *Ehrlichia* polypeptides. Rikihisa does not teach or suggest any isolated 20 amino acid long *Ehrlichia* polypeptides and therefore cannot anticipate claims 1-13.

The examiner's answer also asserts that the declaration of Dr. Chandrashekar does not provide a comparison of proteins of the prior art and the claimed polypeptides and concludes that the declaration is not sufficient to overcome the rejection. The declaration and specification do indeed provide a comparison between the use of whole, partially purified *Ehrlichia* proteins and the purified polypeptides of SEQ ID NOs:1 and 2. The specification demonstrates that in the testing of 70 samples, the polypeptides of SEQ ID NO:1 and SEQ ID NO:2 exhibited a sensitivity of 98.5% and a specificity of 100% in contrast to the whole, partially purified *Ehrlichia* proteins, which exhibited a sensitivity of 75.3% and a specificity of only 60%. See specification, paragraph spanning page 20

and 21. The declaration and specification therefore provide a comparison of proteins of the prior art and the claimed polypeptides.

#### **Claims 10-13**

Claims 10-13 specifically state that the claimed polypeptides are 20 amino acids in length. As such, the claims certainly do not read on whole *Ehrlichia* proteins. Claims 10-13 encompass a 20 amino acid long polypeptide comprising SEQ ID NO:1 and phenotypically silent or conservative amino acid substitution variants thereof. As described above for claims 1-13, Rikihisa does not teach or suggest any polypeptides that are 20 amino acids in length. Those arguments are repeated herein in their entirety.

Rikihisa does not anticipate claims 1-13 because Rikihisa does not teach, suggest, or inherently disclose each and every element of claims 1-13. Applicants respectfully request withdrawal of the rejection.

#### **IV. Claims 1-9 are definite under 35 U.S.C. §112, second paragraph.**

Claims 1-9 stand rejected under 35 U.S.C. §112, second paragraph as allegedly lacking definiteness. Applicants respectfully traverse the rejection.

The Office Action asserts that claims 1-9 are indefinite for the use of the term “phenotypically silent amino acid substitution variants.” Initially, claims 7, 8 and 9 do not contain this term. As such, the rejection is improper for these claims. The Office Action asserts that the metes and bounds of “phenotypically silent amino acid substitutions” cannot be ascertained by what is disclosed in the specification and Bowie *et al.* (of record). However, the test for definiteness is whether “those skilled in the art would understand what is claimed when the claim is read in light of the specification.”

*Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 1 U.S.P.Q.2d 1081, 1088 (Fed. Cir.1986).

Bowie (of record) discusses a research paper that studied the effects of “approximately 1,500 single amino acid substitutions at 142 positions in the lac repressor...[and] found that about one-half of all substitutions were phenotypically silent.” See Bowie, page 1306, right column, first paragraph. Appellants assert that since phenotypically silent amino acid substitutions were described in an article appearing in *Science*, which is one of the most highly regarded scientific peer-reviewed journals, without reference to a definition, one of skill in the art would understand what is claimed. Furthermore, the specification teaches that:

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie *et al.*, *Science*, 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, the amino acid positions which have been conserved between species can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions in which substitutions have been tolerated by natural selection indicate positions which are not critical for protein function. Thus, positions tolerating amino acid substitution may be modified while still maintaining specific binding activity of the polypeptide to anti-*Ehrlichia* antibodies or antibody fragments. See page 7, line 14 through page 8, line 2 (emphasis added).

Therefore, the specification teaches that a phenotypically silent amino acid substitution is an amino acid substitution that allows the variant to maintain specific binding activity of the polypeptide to anti-*Ehrlichia* antibodies or antibody fragments.

One of skill in the art would understand what is claimed in view of the knowledge one of ordinary skill in the art and the teachings of the specification.

The claims are therefore definite and applicants respectfully request withdrawal of the rejection.

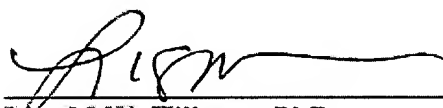
**Summary**

Applicants respectfully submit that the claims are in a condition for allowance.

Respectfully submitted,

Date: 3/28/05

by:

  
\_\_\_\_\_  
Lisa M.W. Hillman, PhD  
Reg. No. 43,673

## APPENDIX A

### CLAIMS AS PENDING

1. (Previously Presented) A composition of matter comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
2. (Previously Presented) The composition of claim 1, further comprising a carrier.
3. (Previously Presented) An article of manufacture comprising packaging material and, contained within the packaging material, a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
4. (Previously Presented) The article of manufacture of claim 3 wherein the packaging material comprises a label that indicates that the polypeptide can be used for the identification of *Ehrlichia* infection in a mammal.
5. (Previously Presented) The article of manufacture of claim 4, wherein the label indicates that identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:
  - (a) contacting a polypeptide the polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant of the polypeptide consisting essentially of SEQ ID NO:2 that specifically binds to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form; and
  - (b) detecting polypeptide/antibody complexes;wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present.

6. (Previously Presented) The article of manufacture of claim 4, wherein the *Ehrlichia* infection is caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*.

7. (Previously Presented) A composition of matter comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.

8. (Previously Presented) An article of manufacture comprising packaging material and, contained within the packaging material, a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.

9. (Previously Presented) The article of manufacture of claim 4, wherein the label indicates that identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

(a) contacting a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant of the polypeptide consisting essentially of SEQ ID NO:2 that specifically binds to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form; and

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present.

10. (Previously Presented) A composition of matter comprising an isolated polypeptide that is 20 amino acids in length, which comprises SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.

11. (Previously Presented) The composition of claim 10, further comprising a carrier.

12. (Previously Presented) A composition of matter comprising an isolated polypeptide that is 20 amino acids in length, which comprises SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.

13. (Previously Presented) The composition of claim 12, further comprising a carrier.

## **APPENDIX B**

A copy of a declaration signed by Dr. Chandrasheka and submitted under Rule 1.132 is attached. The declaration was entered into the record on February 23, 2004.

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UNITED STATES PATENT AND TRADEMARK OFFICE  
(Case No. 00-1278)

In the Application of: )  
)  
Lawton, et al. )  
) Art Unit: 1645  
Serial No.: 09/765,739 )  
) Examiner: V. Ford  
Filed: January 18, 2001 )  
)  
For: Compositions and Methods for Detection of *Ehrlichia canis* and *Ehrlichia chaffeensis* Antibodies

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

1. I, Ramaswamy Chandrashekar, am a research scientist for IDEXX Laboratories, Inc., the entire Assignee of U.S. Pat. Appl. Ser. Nos. 09/765,739, 10/054,354, and 10/054,647. I have earned a Ph.D. in Biochemistry and M.S. in Zoology. I have performed research and development in the field of sero-diagnostics for veterinary bacterial pathogens, including, for example, *Ehrlichia* ssp. and *Streptococcus equi* for over five years. In addition, I have performed research and development in the field of sero-diagnostics for nematode infections in both humans and animals for over 20 years. I am an author of over 70 scientific publications in the field of diagnosis and prevention of nematode infections. A copy of my Curriculum Vitae is attached.

2. Waner *et al.*, (*J. Vet. Diagn. Invest.*, Vol. 12, pp. 240-244, 2000), Cadman *et al.*, (*The Veterinary Record*, 135, 362), Rikihisa (WO 99/13720), and other references

that teach or suggest the use of entire *E. canis* or *E. chaffeensis* infected cells, whole (i.e., non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins, or whole recombinant proteins, to, e.g., detect *Ehrlichia*, do not teach or suggest polypeptides of SEQ ID NOs:1-7 to, e.g., detect *Ehrlichia*. As explained in the specifications of the above-mentioned patent applications, entire *E. canis* or *E. chaffeensis* infected cells, or whole (i.e., non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins or whole recombinant proteins are impure reagents, which are of limited usefulness in sero-diagnosis due to sensitivity and specificity issues. For instance, Example 1 of the 09/765,739 application demonstrates that assays that use SEQ ID NOs:1 and 2 were more sensitive and specific than assays that use partially purified *E. canis* antigens. See e.g., paragraph spanning page 20 and 21 of the 09/765,739 application. The partially purified *E. canis* antigens were obtained from *E. canis* organisms grown in tissue culture and partially purified by differential centrifugation and column chromatography. These partially purified *E. canis* antigens were therefore, mixtures of whole proteins.

3. Assays for detecting anti-*Ehrlichia* antibodies or fragments as described by Waner, Cadman, Rikihisa, and others are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. See e.g., page 2, line 21 through page 3, line 2 of the 09/765,739 specification (emphasis added). The instant inventions provide highly purified reagents for the detection *Ehrlichia*, that is, polypeptides of about 18-20 amino acids. The use of SEQ ID NOs:1-7 instead of the impure reagents described above, to for

example, detect *Ehrlichia* provide distinct advantages such as greater sensitivity and specificity in sero-diagnostic assays.

4. Waner teaches that the disclosed ELISA assay for detection of *E. canis* closely correlates to the "gold standard" IFA test. See e.g., p. 243, left col. first full paragraph; page 243, right col., first and second paragraph; page 240, right col., first full paragraph.

5. Cadman teaches that the disclosed dot-blot enzyme linked immunoassay (DBELIA) had a sensitivity of 92% and a specificity of 96% when compared to the IFA. See page 135, paragraph spanning columns. Cadman states that the "study showed the DBELIA to be as sensitive and specific as IFA for the detection of antibodies to *E. canis*." See last paragraph.

6. The polypeptides claimed in the instant application have a sensitivity of 98.5% and a specificity of 100% when compared to western blot analysis. Western blot analysis is more sensitive and more specific than IFA analysis. The IFA had, at one time, been considered the "gold standard" for sero-diagnosis of *Ehrlichia*. However, western analysis is more sensitive and more specific than IFA analysis, which uses whole cells as the antigen resulting in cross-reactivity, specificity, and sensitivity issues. The IFA disclosed in the instant invention had a sensitivity of 88% and a specificity of 0%. The polypeptides of the instant invention perform better than the "gold standard" IFA in this study. The Waner and Cadman assays, however, perform only as well as the IFA. Therefore, one of skill in the art could reasonably conclude that the polypeptides of the instant invention perform better, i.e., provide more sensitive and specific results in sero-diagnostic assays, than the Waner and Cadman assays.

7. Rikihisa teaches the use of recombinant, whole proteins to detect *Ehrlichia* antibodies. Rikihisa does not disclose the sensitivity or specificity of the whole, recombinant proteins in sero-diagnostic assays. However, Ohashi *et al.* (J. Clin. Microbiol. 36:2671 (1998)) (copy attached) teaches that dot blot assays performed with whole *E. canis* rP30 antigen to detect *E. canis* were as sensitive as an IFA assay, specificity was not examined in this study. See page 2678, right column, first full paragraph. The instant invention provides peptides (SEQ ID NOs:1-7) that can provide results that are more sensitive than IFA assays. Therefore, one of skill in the art could reasonably conclude that the peptides of the instant invention are more sensitive and more specific than the antigens reported in Waner, Cadman, and are more sensitive than the antigens reported in Rikihisa.


8. The pure reagents described in the instant inventions have additional advantages as compared to the impure reagents described in Waner, Cadman, and Rikihisa. For example, in experiments performed at IDEXX Laboratories mixtures of SEQ ID NOs:1 and 2 showed no cross-reactivity to *Borrelia burgdorferi*, *A. phagocytophilum*, and uninfected canine serum. See Table 1.

**Table 1.**

Peptide	# of Samples	Canine Serum	Reactivity
Mixtures of SEQ ID NO:1 and SEQ ID NO:2	157	Uninfected	0/157
	81	<i>E. canis</i>	81/81
	166	<i>Borrelia burgdorferi</i>	0/166
	29	<i>A. phagocytophilum</i>	0/29

9. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 02/13/2004

By:   
Ramaswamy Chandrashekar

Responsible for immunodiagnostic assay development and vaccine discovery studies in parasitic nematode infections. Field evaluation of diagnostic tests in Egypt. Supervised science technicians.

**ACCOMPLISHMENTS**

- Developed recombinant antigen-based antibody ELISAs for the diagnosis of human nematode infections. Evaluation of both assays extensively in field studies in Africa.
- Developed a monoclonal antibody-based antigen-based diagnostic assay for human onchocerciasis (river blindness) to detect circulating parasite antigens both in blood and urine.
- Developed a recombinant antigen-based immunoblot assay for diagnosis of Histoplasmosis.
- Identified and tested several candidate recombinant vaccine antigens in animal model of filarial nematode infections.
- One Patent issued and one pending.

**NATIONAL/INTERNATIONAL SCIENTIFIC ACTIVITIES**

- Invited Participant-WHO: Filariasis Scientific Working Group (UNDP/World Bank/World Health Organization), 1991, 1994. "Protective immunity studies in Onchocerciasis.
- Chairperson: Chaired the scientific session in Filarial Biology. Joint Annual Meeting of the American Society of Tropical Medicine and Hygiene and The American Society of Parasitologists, Atlanta, Georgia. October 31-November 4, 1993.
- Advisor-WHO: Special Program for Research & Training in Tropical Diseases (TDR), World Health Organization, 1993, 1994. Transferred technology from lab research to product development.
- Consultant and collaborating scientist, Epidemiology and Control of Vector Borne Diseases in the Middle East (Egypt-Israel-USA) (USAID/NIH), 1990-1994. Transferred immunodiagnostic assays for lymphatic filariasis from laboratory to field for evaluation studies in Egypt.
- Collaborating Scientist, Participated in protective immunity studies in human filariasis-*International Collaborations in Infectious Disease Research Project (NIH)* to study immunity to filariasis in humans with a longitudinal study of carefully defined populations in a highly endemic region of Egypt, 1994-1995.

**POST-DOCTORAL RESEARCH ASSOCIATE (Jewish Hospital of St. Louis at Washington University) (1988-1991)**

Identified, characterized and generated monoclonal antibodies to circulating parasitic nematode antigens; Developed antigen detection and recombinant antigen-based antibody assays for human infectious diseases. Participated in a Recombinant DNA Technology Workshop conducted by the *New England Biolabs* and Smith College, Northampton (1991). Supervised a science technician.

**CIBA-GEIGY RESEARCH CENTER, BOMBAY, INDIA**

SENIOR RESEARCH ASSISTANT (1986-1988)

RESEARCH ASSISTANT (1980-1986)

**EDUCATION**

Ph.D., Biochemistry - CIBA-GEIGY Research Center, and University of Bombay, India.

MS., Zoology - University of Madras, Madras, India.

**HONORS AND AWARDS**

National Science Talent Search Scholarship, NCERT, New Delhi, India.

Joshua Gold Medal for best under graduate student.

Joshua Gold Medal and Aaron award for best post graduate student.

National Merit Scholarship, Government of India.

**PROFESSIONAL MEMBERSHIPS**

American Society of Tropical Medicine and Hygiene.

American Association for the Advancement of Science.

American Society of Parasitologists.

American Society for Microbiologists.

**PUBLICATIONS**

(SEE ADDENDUM)

Peer-reviewed-64; Invited-7

## ADDENDUM

## PUBLICATIONS

## PEER-REVIEWED

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#### INVITED PUBLICATIONS

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2. Chandrashekar R. Recent advances in the diagnosis of filarial infections. *Ind J Exp Biology*. 1997; 35:18.
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4. Chandrashekar R, Mehta K. Transglutaminase-catalyzed reactions in the growth, maturation, and development of parasitic nematodes. *Parasitology Today*. 2000; 11-17.
5. Chandrashekar R. Molecular Mechanisms of immune evasion by *Dirofilaria immitis*. Heartworm Vaccine and Immunology Research. In: *Recent Advances in Heartworm Disease: Symposium '01*. Ed. Seward RL. American Heartworm Society, Batavia, IL. 2001 (In Press).
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## Cloning and Characterization of Multigenes Encoding the Immunodominant 30-Kilodalton Major Outer Membrane Proteins of *Ehrlichia canis* and Application of the Recombinant Protein for Serodiagnosis

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A 30-kDa major outer membrane protein of *Ehrlichia canis*, the agent of canine ehrlichiosis, is the major antigen recognized by both naturally and experimentally infected dog sera. The protein cross-reacts with a serum against a recombinant 28-kDa protein (rP28), one of the outer membrane proteins of a gene (*omp-1*) family of *Ehrlichia chaffeensis*. Two DNA fragments of *E. canis* were amplified by PCR with two primer pairs based on the sequences of *E. chaffeensis omp-1* genes, cloned, and sequenced. Each fragment contained a partial 30-kDa protein gene of *E. canis*. Genomic Southern blot analysis with the partial gene probes revealed the presence of multiple copies of these genes in the *E. canis* genome. Three copies of the entire gene (*p30*, *p30-1*, and *p30a*) were cloned and sequenced from the *E. canis* genomic DNA. The open reading frames of the two copies (*p30* and *p30-1*) were tandemly arranged with an intergenic space. The three copies were similar but not identical and contained a semivariable region and three hypervariable regions in the protein molecules. The following genes homologous to three *E. canis* 30-kDa protein genes and the *E. chaffeensis omp-1* family were identified in the closely related rickettsiae: *wsp* from *Wolbachia* sp., *p44* from the agent of human granulocytic ehrlichiosis, *msh-2* and *msh-4* from *Anaplasma marginale*, and *map-1* from *Cowdria ruminantium*. Phylogenetic analysis among the three *E. canis* 30-kDa proteins and the major surface proteins of the rickettsiae revealed that these proteins are divided into four clusters and the two *E. canis* 30-kDa proteins are closely related but that the third 30-kDa protein is not. The *p30* gene was expressed as a fusion protein, and the antibody to the recombinant protein (rP30) was raised in a mouse. The antibody reacted with rP30 and a 30-kDa protein of purified *E. canis*. Twenty-nine indirect fluorescent antibody (IFA)-positive dog plasma specimens strongly recognized the rP30 of *E. canis*. To evaluate whether the rP30 is a suitable antigen for serodiagnosis of canine ehrlichiosis, the immunoreactions between rP30 and the whole purified *E. canis* antigen were compared in the dot immunoblot assay. Dot reactions of both antigens with IFA-positive dog plasma specimens were clearly distinguishable by the naked eye from those with IFA-negative plasma specimens. By densitometry with a total of 42 IFA-positive and -negative plasma specimens, both antigens produced results similar in sensitivity and specificity. These findings suggest that the rP30 antigen provides a simple, consistent, and rapid serodiagnosis for canine ehrlichiosis. Cloning of multigenes encoding the 30-kDa major outer membrane proteins of *E. canis* will greatly facilitate understanding pathogenesis and immunologic study of canine ehrlichiosis and provide a useful tool for phylogenetic analysis.

Canine ehrlichiosis is caused by *Ehrlichia canis*, an obligatory intracellular bacterium. It was described originally in Algeria in 1935 (7), and it has now been reported throughout the world and at higher frequency in tropical and subtropical regions (13, 15, 32). Canine ehrlichiosis is characterized by fever, depression, anorexia, and weight loss in the acute phase, with laboratory findings of thrombocytopenia and hypergammaglobulinemia (3, 9). A subclinical phase follows the acute phase (5, 12, 28). In the chronic phase, in addition to the clinical signs and laboratory findings of the acute phase, hemorrhages, epistaxis, edema, and hypotensive shock may occur, which are often exacerbated by superinfection with other organisms (3, 9, 16).

Among several protein antigens of *E. canis*, the proteins in the 30-kDa range were shown to be dominant antigens and

consistently recognized by sera from both experimentally and naturally infected dogs in Western blot analysis (14, 25, 26). The proteins of *E. canis* immunologically cross-react with *Ehrlichia chaffeensis* major antigens in the 30-kDa range (25). These *E. canis* and *E. chaffeensis* proteins were found to be major outer membrane proteins (OMPs) (22). Analysis of a 28-kDa major OMP (P28) gene of *E. chaffeensis*, one of the 30-kDa-range antigens, and its gene copies revealed that these proteins are encoded by a polymorphic multigene family (22). The rabbit serum against a recombinant *E. chaffeensis* P28 protein cross-reacted with the 30-kDa protein of *E. canis* (22).

Dot immunoblot assaying has been developed for serodiagnosis of several infectious agents (4, 10, 11, 30). The advantages of the assay are that an expensive instrument is not required and the interpretation of the results is easy, since positive and negative reactions can be distinguished by the naked eye. However, to be used as the antigen, purification of the organism from infected cells is essential, since *E. canis* is an obligate intracellular bacterium. Purification of *E. canis* is time-consuming and expensive, and serial passages of *E. canis*

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in the cell culture may produce batch-to-batch variations. Although, no genes of *E. canis* other than the 16S rRNA gene have thus far been identified, preparation of a recombinant major antigen is expected to greatly improve the serodiagnosis of *E. canis* infection.

In this study, three genes encoding the 30-kDa OMPs from the *E. canis* genome were identified. All were found to be homologous and phylogenetically characterized. A recombinant protein of *E. canis* which was expressed as a fusion protein was found to be highly antigenic. The dot immunoblot assay was developed with the recombinant *E. canis* protein.

#### MATERIALS AND METHODS

**Organisms and purification.** *E. canis* Oklahoma and *E. chaffeensis* Arkansas were cultivated in the DH82 dog macrophage cell line and purified by Percoll density gradient centrifugation (22) or Sephacryl S-1000 column chromatography (26).

**PCR, cloning, and expression.** The sequences of two forward primers, FECH1 and FECH2, were 5'-GGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG(A/T/G/C)AA(T/C)TT(T/C)TA-3' and 5'-GGGATCCGAATTCCTA(T/C)AT(A/T)AG(T/C)GG(A/T/G/C)AA(A/G)TA(T/C)ATG-3', corresponding to amino acid positions 6 to 12 and positions 12 to 18, respectively, of the mature 28-kDa protein (P28) of *E. chaffeensis* (22). These primers have a 14-bp sequence (underlined) at the 5' end to create an *EcoRI* site and a *BamHI* site for insertion into an expression vector. The sequence of a reverse primer, REC1, was 5'-ACCTAATCTTCCTGGTAA-3', complementary to the DNA sequence corresponding to amino acid positions 185 to 191 of the mature P28 of *E. chaffeensis* (22).

Genomic DNA of *E. canis* was isolated from Percoll gradient-purified organisms as described elsewhere (22). PCR amplification was performed by using a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). The 0.6-kb products were amplified with both primer pairs, FECH1-REC1 and FECH2-REC1, and were cloned in the pCR11 vector of a TA cloning kit (Invitrogen Co., San Diego, Calif.). The clones obtained by FECH1-REC1 and FECH2-REC1 were designated pCR11p30 and pCR11p30a, respectively. Both strands of the insert DNA were sequenced by a dideoxy termination method with an Applied Biosystems 373 DNA sequencer.

For expression, the 0.6-kb fragment was excised from the clone pCR11p30 by *EcoRI* digestion, ligated into the *EcoRI* site of a pET29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLys (Novagen, Inc., Madison, Wis.). The clone (designated pET29p30) produced a fusion protein with 35-amino-acid and 21-amino-acid sequences carried from the vector at the N and C termini, respectively.

For purification of a recombinant P30 fusion protein (rP30), the cultivated clone was harvested at 4 h after induction with  $\beta$ -D-thiogalactopyranoside. The recombinant protein in the clone pET29p30 was enriched in the pellet by three cycles of centrifugation of the lysate after disruption of the transformant by freezing-thawing and sonication. The final pellet was used as a partially purified rP30 antigen. Affinity-purified rP30 protein was obtained by chromatography with His-Bind Resin (Novagen, Inc.). Briefly, after preparation of the partially purified rP30 antigen, the insoluble protein was extracted with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), including 6 M urea. After being applied to a Ni<sup>2+</sup>-conjugated column, the recombinant protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The refolding of the purified protein was achieved by sequential dialysis in 20 mM Tris-HCl (pH 7.9) containing 4 and 2 M urea and finally in 20 mM Tris-HCl buffer only and stored at -80°C until use.

**Southern blot analysis.** Genomic DNA extracted from the Percoll-purified *E. canis* (200 ng each) was digested with restriction enzymes, electrophoresed, and transferred to a Hybond-N<sup>+</sup> nylon membrane (Amersham, Arlington Heights, Ill.) by a standard method (27). The 0.6-kb DNA inserts containing partial p30 and p30a genes, cloned in pCR11p30 and pCR11p30a, respectively, were separately labeled with [ $\alpha$ -<sup>32</sup>P]dATP by the random primer method with a kit (Amersham), and each labeled fragment was used for Southern blot analysis as a DNA probe. Hybridization was performed at 60°C in Rapid Hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1× SSC (1× SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate) with 1% sodium dodecyl sulfate (SDS) at 55°C, and the hybridized probes were exposed to Hyperfilm (Amersham) at -80°C.

**Cloning and sequencing of 30-kDa protein gene copies from the *E. canis* genome DNA.** The *HindIII* DNA fragment, which was detected by genomic Southern blot analysis as described above, was inserted into pBluescript II KS(+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5a. By using the colony hybridization method (27), two positive clones which contained chloramphenicol DNA fragments of 3.6 and 7.3 kb were isolated with the <sup>32</sup>P-labeled inserts of pCR11p30 and pCR11p30a as probes, respectively. DNA sequencing was performed with suitable synthetic primers by the dideoxy termination method described above.



FIG. 1. Genomic Southern blot analysis of *E. canis* DNA with the partial p30 gene probe (A) and with the partial p30a gene probe (B) and schematic representation of the blotting patterns (C). Numbers indicate molecular sizes in kilobases. Filled dots, bands hybridized with both p30 and p30a probes; striped dots, bands hybridized with p30a probe alone; lightly shaded dots, bands hybridized with p30 probe alone.

**Sequence analysis.** DNA and amino acid sequences were analyzed with the programs DNASIS (Hitachi Software Engineering America, Ltd., San Bruno, Calif.) and DNASTAR (DNASTAR Inc., Madison, Wis.). The amino acid sequences were aligned by using the CLUSTAL method in the DNASTAR program. Phylogenetic analysis was performed by using the PHYLIP software package (version 3.5) (8). An evolutionary distance matrix, generated by using the Kimura formula in the program PROTDIST in the package, was used for construction of a phylogenetic tree by using the unweighted pair-group method of analysis (8). The data were examined by using parsimony analysis (PROTPARS in the PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package.

**Dog plasma and mouse serum.** Totals of 34 and 8 dog blood samples with heparin or EDTA were obtained from the Southwest Veterinary Diagnostic Center (Phoenix, Ariz.) and at the Ohio State University Veterinary Teaching Hospital, respectively. All blood specimens collected were centrifuged at 250 × g for 5 min, and the plasma samples were used for this study. For Western blot analysis, these plasma samples were preabsorbed three times with pET29a-transformed *E. coli* at 4°C overnight prior to use. For preparation of the mouse anti-rP30 serum, a male mouse (BALB/c) was intraperitoneally immunized a total of four times at 10-day intervals, once with an equal mixture of the affinity-purified rP30 (30 µg of protein) and Freund's complete adjuvant (Sigma) and three times with an equal mixture of the protein (30 µg) and Freund's incomplete adjuvant. The mouse was sacrificed 7 days after final immunization, and the serum was prepared from blood collected from the heart.

**IFA and Western blot analysis.** Indirect fluorescent antibody assays, (IFA) and Western blot analysis were performed by a procedure described elsewhere (25). Fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (IgG; Organon Teknica Co., Durham, N.C.) and peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were used at dilutions of 1:200 for IFA and 1:2,000 for Western blot analysis, respectively, as secondary antibodies.

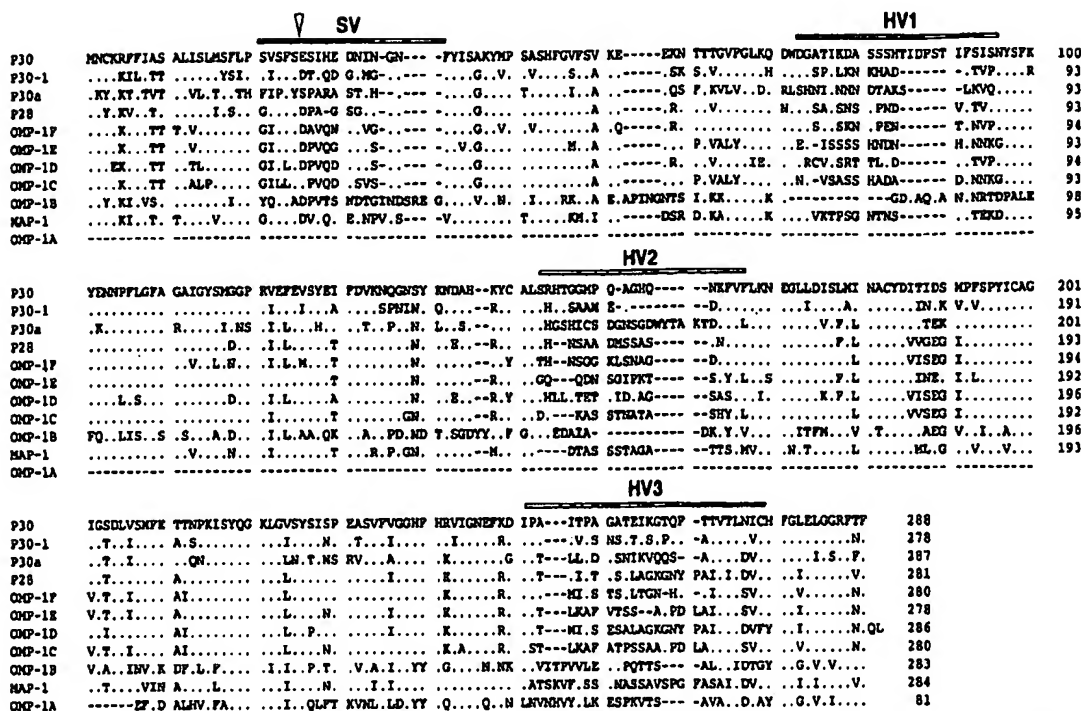


FIG. 2. Amino acid sequence alignment of P30, P30-1, and P30a of *E. canis*, seven members of *E. chaffeensis* *omp-1* multigene family (P28 and OMP-1A to OMP-1F), and MAP-1 of *C. ruminantium* (Senegal strain). The sequences of the *E. chaffeensis* *omp-1* gene family and MAP-1 are from the reports of Ohashi et al. (22) and Van Vliet et al. (31), respectively. Aligned positions of identical amino acids with P30 of *E. canis* are indicated by dots. Gaps (indicated by dashes) were introduced for optimal alignment of all proteins. Bars indicate an SV and three HVs (HV1, -2, and -3). The arrowhead indicates the putative cleavage site of the signal peptide.

Dot immunoblot assay. Protein concentrations of purified *E. canis* and recombinant rP30 antigens were determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard. These antigens in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) were adsorbed onto a nitrocellulose membrane by using a dot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), blocked for 30 min with TBS containing 2% milk, air dried, and stored at  $-20^{\circ}\text{C}$  until use. For immunoassays, the antigen bound to a nitrocellulose strip was incubated with the plasma samples, which were diluted 1:1,000 in TBS containing 2% milk for 1 h at room temperature. After being washed three times with TBS containing 0.05% Tween 20 (T-TBS),

the strip was incubated with peroxidase-conjugated affinity-purified anti-dog IgG (Kiergaard) at a dilution of 1:2,000 in TBS containing 2% milk. After being washed with T-TBS, the antibody-bound dot was detected by immersing the strip in a developing solution (0.3% 3,3'-diaminobenzidine tetrahydrochloride [Nacalai Tesque, Inc., Kyoto, Japan] and 0.05% hydrogen peroxide in 70 mM sodium acetate [pH 6.2]). The color intensity was analyzed by using background correction in image analysis software (ImageQuant program; Molecular Dynamics, Sunnyvale, Calif.).

GenBank accession number. The DNA sequences of the *p30*, *p30a*, and *p30-1* genes of *E. canis* have been assigned GenBank accession numbers AF078553, AF078555, and AF078554, respectively.

## RESULTS

### Cloning and sequencing of three 30-kDa protein gene copies of *E. canis*. Two 0.6-kb DNA fragments containing partial *p30*

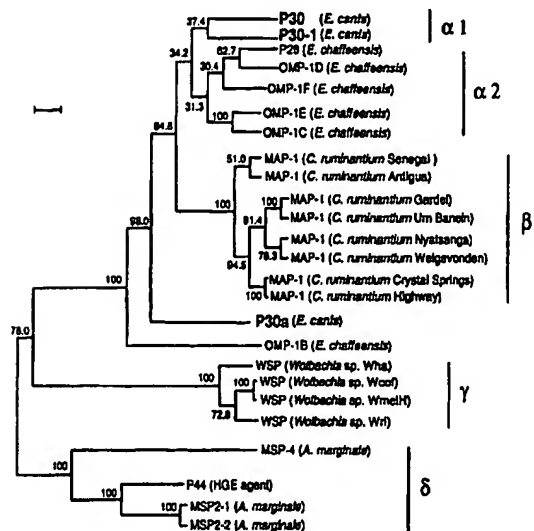


FIG. 3. Phylogenetic classification among P30, P30-1, and P30a of *E. canis* and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Evolutionary distance values were determined by the method described by Kimura, and the tree was constructed by the unweighted pair-group method of analysis. Scale bar indicates 10% divergence in amino acid sequences. Bootstrap values from 100 analyses are shown at the branch points of the tree. Bars with symbols indicate representative clusters. The GenBank accession numbers of the major OMP gene sequences of the organisms used in the analysis are as follows: P28 (*E. chaffeensis*), U72291; OMP-1B to OMP-1F (*E. chaffeensis*), AF021338; MAP-1 (*C. ruminantium* Senegal strain), U08882; MAP-1 (*C. ruminantium* Antigua strain), U50830; MAP-1 (*C. ruminantium* Gardel strain), U50832; MAP-1 (*C. ruminantium* Um Bancein strain), U50835; MAP-1 (*C. ruminantium* Nyatsanga strain), U50834; MAP-1 (*C. ruminantium* Welgevonden strain), U49843; MAP-1 (*C. ruminantium* Crystal Springs strain), U50831; MAP-1 (*C. ruminantium* Highway strain), U50833; WSP (*Wolbachia* sp. Wha strain), AF020068; WSP (*Wolbachia* sp. Wcof strain), AF020067; WSP (*Wolbachia* sp. WmelH strain), AF020066; WSP (*Wolbachia* sp. Wri strain), AF020070; MSP-4 (*A. marginale*), Q07408; MSP-2-1 (*A. marginale*), U07862; MSP-2-2 (*A. marginale*), U36193; and P44 (HGE agent), AF059181.

TABLE 1. Similarities among amino acid sequences of *E. canis* P30, P30-1, and P30a; *E. chaffeensis* omp-1 family (OMP-1B to OMP-1F and P28); *C. ruminantium* MAP-1; *Wolbachia* spp. WSP; HGE agent P44; and *A. marginale* MSP-4, MSP-2-1, and MSP-2-2

Protein	% Amino acid sequence similarity and evolutionary distance for the following proteins <sup>a</sup> :										
	P30	P30-1	P30a	P28	OMP-1F	OMP-1E	OMP-1D	OMP-1C	OMP-1B	MAP-1 (Senegal)	MAP-1 (Antigua)
P30		80.2	70.8	80.6	80.5	78.6	77.8	77.5	63.2	75.4	76.2
P30-1	0.38628		71.6	79.8	81.7	78.7	78.3	77.3	63.2	74.7	75.6
P30a	0.60811	0.60559		73.9	72.1	73.3	71.2	72.1	58.8	67.2	67.8
P28	0.36288	0.40582	0.50899		85.7	82.3	86.3	81.1	63.6	76.4	77.5
OMP-1F	0.37862	0.36209	0.59907	0.27551		83.4	84.9	83.0	63.2	75.4	75.8
OMP-1E	0.41426	0.42866	0.52142	0.35465	0.32640		81.7	90.1	63.4	76.8	78.1
OMP-1D	0.45193	0.46724	0.61591	0.25793	0.28867	0.36288		81.5	63.2	73.5	74.5
OMP-1C	0.45426	0.48329	0.57469	0.39823	0.34577	0.18285	0.37688		62.4	76.0	77.5
OMP-1B	0.89214	0.87276	0.99793	0.81397	0.83501	0.82982	0.84498	0.89516		62.7	63.2
MAP-1 (Senegal)	0.50490	0.51605	0.76041	0.46987	0.50383	0.46987	0.57453	0.50564	0.92668		93.9
MAP-1 (Antigua)	0.47614	0.50899	0.74635	0.46755	0.52220	0.46096	0.57153	0.48952	0.88842	0.09122	
MAP-1 (Gardel)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.13499	0.11546
MAP-1 (Crystal Springs)	0.55702	0.53478	0.78883	0.52220	0.56563	0.49693	0.59089	0.53368	0.93601	0.13657	0.14142
MAP-1 (Highway)	0.52891	0.52047	0.76041	0.49443	0.54364	0.46987	0.57594	0.50564	0.93601	0.12383	0.12856
MAP-1 (Nyatsanga)	0.50593	0.49693	0.76544	0.49196	0.53368	0.46755	0.57296	0.48952	0.91855	0.13077	0.11963
MAP-1 (Um Banein)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.12658	0.11963
MAP-1 (Welgevonden)	0.52629	0.50383	0.74708	0.49877	0.53368	0.47419	0.60290	0.48952	0.92979	0.16080	0.14519
WSP (Wha)	1.57097	1.66864	1.78274	1.59949	1.50435	1.38174	1.61950	1.45510	1.41776	1.58338	1.48404
WSP (Wcof)	1.46262	1.62571	1.62571	1.55195	1.40877	1.29961	1.60271	1.41762	1.33110	1.53897	1.53089
WSP (WmelH)	1.48165	1.64952	1.64952	1.54244	1.39991	1.31514	1.59304	1.43572	1.34750	1.54961	1.49206
WSP (Wri)	1.46435	1.66864	1.70518	1.55687	1.46526	1.27219	1.57654	1.39076	1.32111	1.53292	1.47465
P44	1.77884	1.84928	2.04164	1.56146	1.74020	1.64702	1.64376	1.64702	1.64566	1.57894	1.63909
MSP-4	1.37226	1.39399	1.62744	1.38660	1.45473	1.36494	1.45413	1.47002	1.34294	1.23482	1.31702
MSP-2-1	1.50323	1.53992	1.90757	1.40230	1.59474	1.53455	1.40877	1.50435	1.52758	1.53992	1.54847
MSP-2-2	1.52476	1.53992	1.87540	1.40230	1.57132	1.53455	1.40877	1.50435	1.55019	1.51796	1.52616

<sup>a</sup> Values in the upper right half are percent amino acid sequence similarities; those in the lower left half are evolutionary distances.

and *p30a* genes, amplified by PCR, were cloned and sequenced as described in Materials and Methods. The 0.6-kb DNA, cloned in pCRII $\beta$ 30, had an open reading frame (ORF) of 579 bp encoding a 193-amino-acid protein with a molecular mass of 21,175 Da. Another 0.6-kb fragment, cloned in pCRII $\beta$ 30a, had an ORF of 564 bp encoding a 188-amino-acid protein with a molecular mass of 21,042 Da. The DNA and predicted amino acid sequences of the partial *p30a* gene were similar but not identical to those of the partial *p30* gene. Genomic Southern blot analysis of *E. canis* digested with several restriction enzymes revealed one and two DNA fragments which could strongly hybridize to the partial *p30* and *p30a* gene probes, respectively (Fig. 1). These restriction enzymes used do not cut within the *p30* and *p30a* gene probes, and, therefore, the result with the *p30a* probe indicates that another gene homologous to the *p30a* is present in the *E. canis* genome. In *Bgl*II, *Eco*RI, and *Pst*I digestion, the *p30* probe hybridized with the upper band of the two *p30a*-hybridized bands. In *Eco*RV and *Xba*I digestion, the *p30* probe hybridized with the lower band of the two *p30a*-hybridized bands. In *Kpn*I, *Spe*I, and *Hind*III digestion, the *p30* probe hybridized with one or two bands that were different from the *p30a*-hybridized bands.

Two DNA fragments of 3.6 and 7.3 kb were cloned by colony hybridization with the probes described above from the *Hind*III-digested genomic DNA of *E. canis*. Sequencing revealed a complete ORF of 864 bp for the *p30* gene in the 3.6-kb fragment and a complete ORF of 861 bp for *p30a* gene in the 7.3-kb DNA fragment. An additional ORF of 921 bp was found in the 3.6-kb DNA. The DNA sequence of the ORF (designated *p30-1*) was also similar but not identical to those of the *p30* and *p30a* genes. There are two potential start codons in the *p30-1* gene sequence. By comparison with the N-terminal amino acid sequences of *p30* and *p30a* genes, we chose a second ATG as a start codon for phylogenetic analysis. The coding region is 834

bp. The *p30-1* and *p30* genes were tandemly arranged with an intergenic space of 355 bp in the 3.6-kb fragment like the *E. chaffeensis* omp-1 family (22). In addition to the result of the genomic Southern blot analysis, this finding showed that at least four homologous genes (*p30*, *p30-1*, *p30a*, and a gene homologous to *p30a*) exist in the *E. canis* genome, suggesting that these genes of *E. canis* are also encoded by a polymorphic multigene family as is the case with *E. chaffeensis* (22).

**Structure of proteins encoded by *E. canis* multigenes.** Three complete gene copies (*p30*, *p30-1*, and *p30a*) encode 278- to 288-amino-acid proteins with molecular masses of 30,485 to 31,529 Da. The 25-amino-acid sequence at the N termini of *p30*, *p30-1*, and *p30a* (encoded by *p30*, *p30-1*, and *p30a*, respectively) is predicted to be a signal peptide, as described previously (22). The molecular masses of the mature proteins calculated based on the predicted amino acid sequences are 28,750 Da for *p30*, 27,727 Da for *p30-1*, and 29,132 Da for *p30a*.

The predicted amino acid sequences of *E. canis* P30, P30-1, and P30a showed high similarity with those of members in the *E. chaffeensis* omp-1 gene family (22) and that of major antigen protein 1 (MAP-1) of *Cowdria ruminantium* (31). These organisms are also serologically cross-reactive (6, 17, 18, 19, 20). The alignment of amino acid sequences of these proteins revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules (Fig. 2). The significant differences in sequences among the proteins are observed in the regions designated SV (semivariable region) and HV (hypervariable region). Computer analysis for hydropathy revealed that protein molecules predicted for three *E. canis* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of typical transmembrane proteins. HV1 and HV2 were located in the hydrophilic regions (data not shown).

TABLE 1—Continued

% Amino acid sequence similarity and evolutionary distance for the following proteins:													
MAP-1 (Gardel)	MAP-1 (Crystal Springs)	MAP-1 (Highway)	MAP-1 (Nyatsanga)	MAP-1 (Um Banein)	MAP-1 (Welgevonden)	WSP (Wha)	WSP (Wcof)	WSP (WmelH)	WSP (Wri)	P44	MSP-4	MSP2-1	MSP2-2
76.4	74.5	75.4	75.8	76.4	75.2	44.4	44.6	44.4	44.4	19.5	45.6	27.8	27.4
74.7	73.9	74.3	74.7	74.7	74.5	44.0	45.1	44.8	44.6	20.5	47.6	29.3	29.1
67.6	65.9	66.5	66.7	67.6	67.2	41.5	43.2	42.9	42.5	19.5	43.1	24.2	24.2
75.8	74.5	75.4	75.2	75.8	74.9	44.0	44.8	44.8	44.6	22.5	46.9	29.7	29.5
74.5	73.3	73.9	73.9	74.5	73.9	44.6	45.9	45.9	45.3	21.1	46.2	27.8	27.8
76.2	75.4	76.2	76.0	76.2	75.8	45.7	46.9	46.7	46.9	22.0	47.5	28.2	28.0
74.1	73.1	73.5	73.3	74.1	72.4	43.6	44.2	44.2	44.2	22.0	46.0	29.9	29.7
75.8	74.5	75.4	75.6	75.8	75.6	45.3	46.1	45.9	46.1	22.0	46.6	28.6	28.4
63.6	63.2	63.2	63.2	63.6	62.9	45.5	45.1	44.8	45.5	19.1	45.8	26.9	26.5
91.4	90.7	91.4	91.6	91.8	90.1	44.6	45.1	45.1	45.1	21.8	48.8	28.0	28.0
91.8	90.7	91.4	91.6	91.6	90.3	44.8	45.1	45.3	45.3	21.8	48.0	28.0	28.0
	92.2	92.8	94.9	99.6	93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.12928		98.9	93.1	92.4	93.1	43.4	43.4	43.4	43.2	20.0	46.1	26.7	26.7
0.11692	0.01764		93.7	93.1	93.7	43.8	43.8	43.8	43.6	20.2	46.5	27.2	27.2
0.08788	0.11285	0.10076		94.5	95.4	43.8	43.8	43.8	43.8	20.5	46.7	28.0	27.8
0.00693	0.12514	0.11285	0.09570		93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.11966	0.11285	0.10076	0.08014	0.11966		44.2	44.0	44.0	44.0	20.2	46.5	27.8	27.6
1.51972	1.73099	1.65953	1.64538	1.51972	1.58048		86.1		90.3	12.5	42.5	22.9	22.7
1.47157	1.59304	1.53089	1.55897	1.47157	1.52893	0.27243		98.3	90.9	13.6	42.1	24.0	24.0
1.46262	1.58338	1.52153	1.54961	1.46262	1.51972	0.26757	0.03029		90.7	13.6	42.3	23.8	23.8
1.44526	1.64362	1.57654	1.53292	1.44526	1.50279	0.18429	0.17605	0.17691		13.6	43.2	24.0	23.8
1.62813	1.74020	1.71093	1.68253	1.62813	1.71093	2.06354	2.15803	2.14440	2.09032		25.7	45.5	45.2
1.33120	1.35101	1.30992	1.31112	1.33120	1.33120	1.72157	1.96007	1.90199	1.72157	1.20170		35.6	34.9
1.50996	1.57836	1.53304	1.46817	1.50996	1.48884	1.70865	1.79325	1.81891	1.72741	0.83164	1.20880		95.6
1.50996	1.55543	1.51116	1.46817	1.50996	1.48884	1.70865	1.75923	1.78382	1.72741	0.84284	1.23930	0.05064	

Phylogenetic relationship among the three *E. canis* 30-kDa proteins and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Recently, several major OMP genes which are closely related to the *E. canis* 30-kDa protein have been cloned from rickettsiae (2, 21–24, 31, 34). The phylogenetic tree consisting of 25 major OMPs of the organisms including P30, P30-1, and P30a of *E. canis* was constructed from the estimated evolutionary distances (Fig. 3). The overall pattern of the tree reflects the result based on 16S rRNA gene sequence analysis of the rickettsiae. The 23 representatives, except for *E. canis* P30a and *E. chaffeensis* OMP-1B, are divided into four groups as follows: *E. canis* and *E. chaffeensis*, group  $\alpha$ ; *C. ruminantium*, group  $\beta$ ; *Wolbachia* sp., group  $\gamma$ ; and the agent of human granulocytic ehrlichiosis (HGE) and *Anaplasma marginale*, group  $\delta$ . Group  $\alpha$  formed a subcluster of *E. canis* P30 and P30-1 (group  $\alpha_1$ ), which was separated from another subcluster composed of five *E. chaffeensis* OMPs (group  $\alpha_2$ ). The similarities between P30 and P30-1 of *E. canis* in group  $\alpha_1$ , between groups  $\alpha_1$  and  $\alpha_2$ , between groups  $\alpha_1$  and  $\beta$ , between groups  $\alpha_1$  and  $\gamma$ , and between groups  $\alpha_1$  and  $\delta$  were 80.2%, 77.3 to 80.6%, 73.9 to 76.4%, 44.0 to 45.1%, and 19.5 to 47.6%, respectively (Table 1). On the other hand, *E. canis* P30a and *E. chaffeensis* OMP-1B were far from group  $\alpha$  and were located between groups  $\beta$  and  $\gamma$ . The similarities between *E. canis* P30a and group  $\alpha_1$ , between P30a and group  $\alpha_2$ , between P30a and group  $\beta$ , between P30a and group  $\gamma$ , and between P30a and group  $\delta$  were 70.8 to 71.6%, 71.2 to 73.9%, 65.9 to 67.8%, 41.5 to 43.2%, and 19.5 to 43.1%, respectively.

Expression of the *E. canis* p30 gene. The clone pET29p30 produced a 249-amino-acid fusion protein with a molecular mass of 27,316 Da (Fig. 4A). The recombinant protein (rP30) with minimum *E. coli* contamination detectable was obtained in the pellet by centrifugation of the lysate of the transformant (Fig. 4B [partially purified antigen]). The rP30 protein further

purified by affinity chromatography from this preparation had a single band on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4B [affinity-purified antigen]). The immunoreactions of *E. canis* rP30 with a total of 42 clinical dog plasma specimens were examined. The IgG-IFA titers of 29 plasma samples were 1:20 to 1:10,480. The remaining plasma samples were IFA negative (<1:20). Western blot analysis revealed that all IFA-positive plasma samples recognized the partially purified rP30 fusion protein (27 kDa) and a 30-kDa protein of

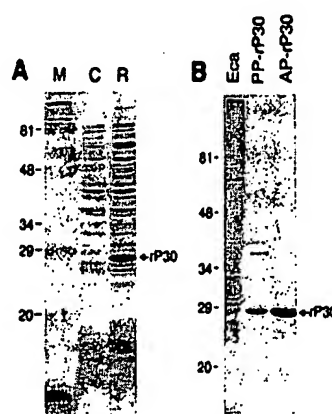


FIG. 4. SDS-PAGE profiles of a recombinant clone expressing P30 of *E. canis* (A) and the purified recombinant protein (B). Gels were stained with Coomassie blue. Lanes: M, molecular size markers; C, pET29-transformed *E. coli* (negative control); R, pET29p30-transformed *E. coli* (recombinant); Eca, purified *E. canis*; PP-rP30, partially purified rP30 fusion protein of *E. canis*; and AP-rP30, affinity-purified rP30 fusion protein. The recombinant rP30 protein is indicated by the arrow. The numbers on the left of each panel indicate molecular masses in kilodaltons.

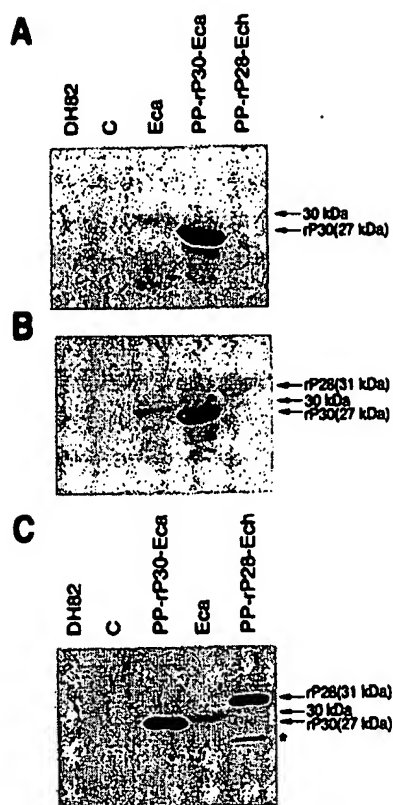


FIG. 5. Western blot analysis with clinical dog plasma with canine ehrlichiosis (A and B) and mouse anti-rP30 serum (C). (A) Dog plasma with a 1:40 IFA titer against *E. canis*; (B) dog plasma with a 1:1,280 IFA titer. Lanes: DH, DH82 dog macrophage cell (negative control); C, a pET29-transformed *E. coli* (negative control); Eca, purified *E. canis* (reactive 30-kDa protein is indicated by arrows in each panel); PP-rP30-Eca, a partially purified rP30 fusion protein (27 kDa) of *E. canis*; and PP-rP28-Ech, a partially purified rP28 fusion protein (31 kDa) of *E. chaffeensis* (22). Another smaller reactive band which may be a degradation product of rP28 of *E. chaffeensis* is indicated by an asterisk.

purified *E. canis* (one of the blots is shown in Fig. 5A), but none of 13 negative plasma samples reacted with any proteins of partially purified rP30 and purified *E. canis* (data not shown). Eight of the 29 positive plasma samples reacted weakly with recombinant P28 fusion protein (rP28 [31 kDa]) of *E. chaffeensis* (22) (one of the blots is shown in Fig. 5B), but the remaining plasma samples did not. A mouse anti-rP30 serum which was prepared by immunization with the affinity-purified antigen reacted with the rP30 antigen, a 30-kDa protein of purified *E. canis*, and an rP28 of *E. chaffeensis* (Fig. 5C). Another smaller band which was observed with *E. chaffeensis* rP28 may be a degradation product of rP28 (asterisk in Fig. 5C), since the plasma sample did not react with *E. coli* proteins. These results showed that rP30 of *E. canis* is highly antigenic and that the antigenic epitope is expressed.

**Dot immunoblot assay with the purified whole organism antigen and the recombinant antigen.** (i) **Optimum amount of antigen per dot.** Western blot analysis and dot immunoblot assaying in the preliminary experiments supported the interpretation that there are no significant differences between affinity-purified and the partially purified rP30 in specificity and sensitivity (data not shown). If partially purified recombinant protein is suitable for serodiagnosis, it will be more cost-effective. By dot immunoblot assaying we examined in detail whether

partially purified rP30 is suitable as an antigen for serodiagnosis.

Nitrocellulose strips having serially diluted purified *E. canis* or partially purified rP30 antigen of *E. canis* were reacted at a 1:1,000 dilution with dog plasma samples with different IFA titers against *E. canis*, and the color intensities of the reaction of each dot were compared (Fig. 6). Dots of 0.01 to 1  $\mu$ g of the purified organisms (Fig. 6A) or dots of 0.025 to 1  $\mu$ g of rP30 (Fig. 6B) that reacted with positive plasma samples ( $>1:20$  in IFA titer) were clearly distinguishable from those that reacted with negative plasma samples ( $<1:20$ ) by the naked eye. There was no nonspecific reaction with the negative plasma samples when purified *E. canis* was used as an antigen; however, a weak nonspecific reaction with IFA-negative plasma was observed in dots of 0.25 to 1  $\mu$ g of partially purified rP30 antigen. Based on these results, the optimum amounts of antigens per dot were determined to be 1 and 0.5  $\mu$ g for antigen proteins of purified *E. canis* and partially purified rP30, respectively. These results show that the partially purified recombinant protein is apparently sufficient as an antigen for serodiagnosis.

(ii) **Optimum dilution of antiserum.** The immunoreactivities of plasma at dilutions of 1:300, 1:1,000, and 1:3,000 were examined with nitrocellulose strips of the purified *E. canis* an-

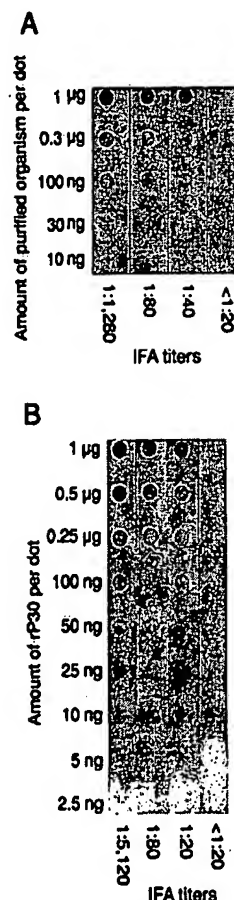


FIG. 6. Optimum amount of antigens for dot blot assaying with purified *E. canis* antigen (A) or partially purified rP30 antigen (B). Purified organism antigen (10 ng to 1  $\mu$ g) or rP30 antigen (2.5 ng to 1  $\mu$ g) was blotted onto the nitrocellulose sheet, reacted with each plasma at a 1:1,000 dilution as primary antibody, and reacted with secondary antibody (peroxidase-conjugated affinity-purified anti-dog IgG antibody) at a 1:2,000 dilution.

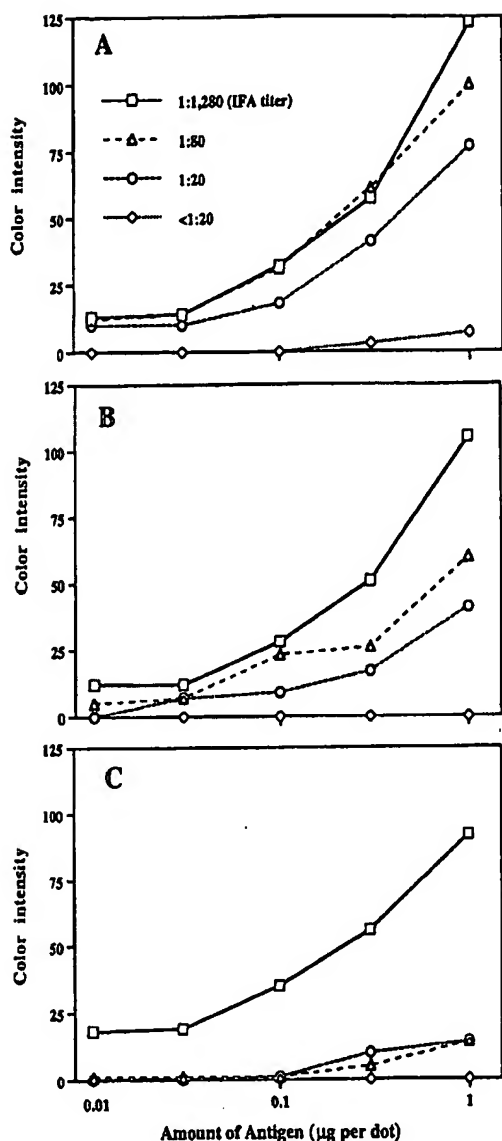


FIG. 7. Optimum plasma dilutions for dot blot assay. Purified *E. canis* antigen was blotted as described in the legend to Fig. 6. The antigens were incubated with plasma at dilutions of 1:300 (A), 1:1,000 (B), and 1:3,000 (C). The plasma samples used were the same as those used for Fig. 6A. The color intensity of each dot was determined by using the image software program (ImageQuant).

tigen as shown in Fig. 6A. The color intensity values were plotted in graphs (Fig. 7). At a 1:300 dilution (Fig. 7A), color development occurred in the dots having an antigen greater than 0.3 µg per dot with IFA-negative plasma. At a 1:3,000 dilution (Fig. 7C), color intensities of all plasma samples were low, especially in the case of positive plasma samples with low IFA titers (1:20 and 1:80). At a 1:1,000 dilution (Fig. 7B), positive plasma with even the lowest IFA titer (1:20) was distinguishable from IFA-negative plasma by the naked eye, especially with 1 µg of purified *E. canis* antigen per dot (Fig. 6A). The optimum dilution of plasma for testing was, therefore, 1:1,000.

(iii) Examination of clinical dog plasma with purified *E. canis* and partially purified rP30 antigens. A total of 42 clinical

dog plasma samples were examined with 1 µg of purified *E. canis* antigen per dot and 0.5 µg of partially purified rP30 antigen per dot (Fig. 8). The plasma samples with higher IFA titers showed a darker reaction with both native and recombinant antigens. The color intensities between plasma with IFA titers of >1:20 and IFA-negative plasma were clearly distinguishable by the naked eye. The correlation between IFA titers and color intensity values by the dot immunoblot assay was examined (Fig. 9). The maximum color intensity values of 13 IFA-negative plasma samples (<1:20) were zero (background) in the purified *E. canis* antigen and 10 in the rP30 antigen. All 29 IFA-positive plasma samples (>1:20) showed color intensity values of greater than 19 in the purified *E. canis* and 18 in the rP30 antigen. The highest color intensity values were 105 in the purified organism and 114 in the rP30 antigen. In both native and recombinant antigens, color intensity values correlated with IFA titers. The correlation coefficients between IFA titers and color intensities of native and recombinant antigens were 0.71 ( $P < 0.001$ ) and 0.68 ( $P < 0.001$ ), respectively. Therefore, it may be possible to estimate an approximate titer of the test serum or plasma by comparing the color densities with those of serially diluted standard serum or plasma.

## DISCUSSION

The availability of recombinant immunodominant major surface proteins of *E. canis* will greatly assist in diagnosis and in understanding of the pathogenesis of this intracellular bacterium, such as invasion of host cells, elicitation of the immune response, and mechanisms of the clinical disease. The 30-kDa protein of *E. canis* was shown to be the immunodominant major OMP, which can be recognized by naturally and experimentally infected dog sera (14, 25, 26). Therefore, the 30-kDa protein is the primary recombinant antigen candidate for use in the serodiagnosis of *E. canis* infection. The present study is the first report of molecular characterization of 30-kDa major OMPs of *E. canis*.

Polymorphic multigene families encoding the major OMPs have been identified in *E. chaffeensis*, the HGE agent, and *A. marginale*, which are closely related to *E. canis* based on 16S rRNA gene sequences. Six copies of the *E. chaffeensis* *p28* gene (*omp-1* gene family) are tandemly arranged with intergenic spaces (22), while copies of the HGE agent *p44* gene and the *A. marginale* *msp-2* and *msp-3* genes are distributed widely throughout the genomes (1, 23, 34). In this study, the 30-kDa proteins of *E. canis* were also shown to be encoded by a polymorphic multigene family. The two *E. canis* genes are tandemly arranged with an intergenic space as are members of the *E. chaffeensis* *omp-1* gene family. Although we demonstrated the presence of four gene copies of 30-kDa *E. canis* proteins in the genome, additional gene copies which are tandemly arranged may exist in three genomic *Hind*III DNA fragments which hybridized to *p30* and *p30a* probes. Sequence analysis revealed that the 30-kDa proteins (P30, P30-1, and P30a) of *E. canis* had characteristics of the *E. chaffeensis* OMP-1 family (22) and *C. ruminantium* MAP-1 (31). The *C. ruminantium* MAP-1 has been reported to be cross-reactive to a 27-kDa protein of *E. canis* (19), although it is unknown whether the 27-kDa protein is identical to P30, P30-1, or P30a of *E. canis* in this study. Phylogenetic analysis based on the homologs from the closely related rickettsiae revealed that P30 and P30-1 of *E. canis* are present in the same cluster but that P30a is far from the cluster, suggesting that the multigenes encoding the 30-kDa *E. canis* proteins are widely divergent. Interestingly, in the phylogenetic tree, the 30-kDa *E. canis* proteins, the *E. chaffeensis* OMP-1 family, the HGE agent P44, and *A. mar-*

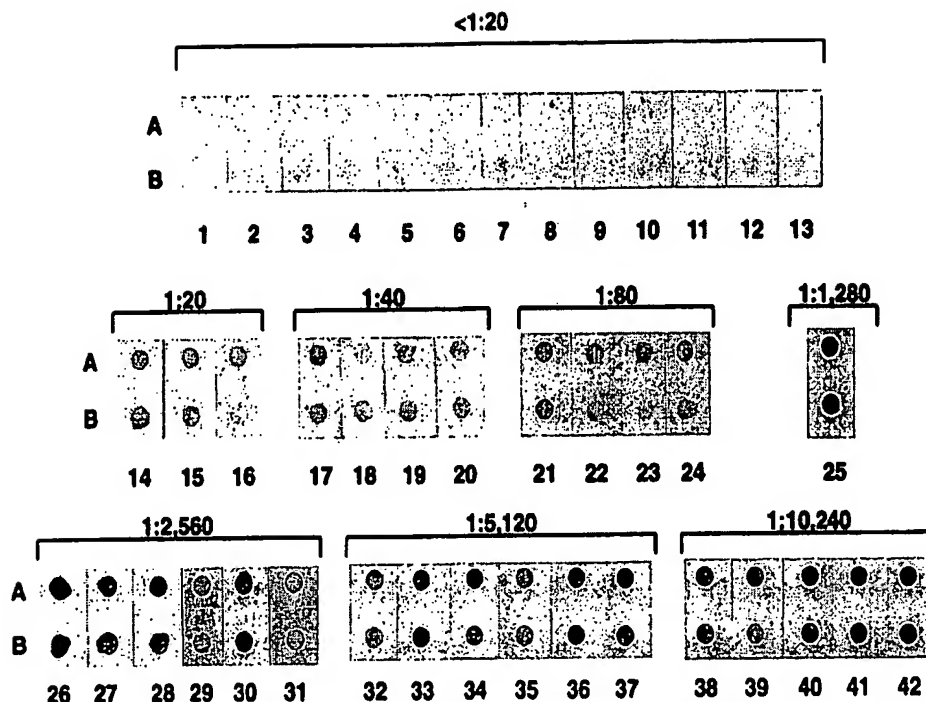


FIG. 8. Reaction profiles of purified *E. canis* antigen (1  $\mu$ g) (A) and partially purified rP30 antigens (0.5  $\mu$ g) (B) with 42 plasma samples. Plasma identifications are indicated below each dot. Numbers above brackets indicate the IFA titers of the plasma samples.

*ginale* MSP-2 are encoded by a polymorphic multigene family as described above. However, *C. ruminantium* MAP-1, *Wolbachia* sp. WSP, and *A. marginale* MSP-4 are encoded by a single gene (2, 21-24, 31). The diversities reported among the *C. ruminantium* MAP-1s and among the *Wolbachia* sp. WSPs are strain variation (2, 24, 31).

Molecular analysis of *E. canis* 30-kDa antigens such as ours is important in understanding the antibody responses of animals, because the antigenic diversity may influence the specificity and sensitivity of the serologic assay. Previously, we observed in the Western blot analysis that acute-phase serum (before 30 days postinoculation) from an *E. canis*-infected dog reacted strongly with a 30-kDa protein but weakly with a 31-kDa protein. However, the reactivity of the chronic-phase serum (after 60 days postinoculation) from the same dog was reversed (strong reaction with the 31-kDa protein and weak reaction with the 30-kDa protein) (14). This might be due to differential expression of the multigene encoding the 30-kDa protein of *E. canis* during infection. Although it is unknown whether the genes of P30, P30-1, and P30a were expressed by *E. canis* in tissue culture or in the infected dog, the recombinant P30 protein constructed in this study expressed the antigenic epitope which can react with all IFA-positive dog plasma samples used, suggesting that the antigenic epitope conserved among the 30-kDa protein gene family is expressed. This strongly supports the idea that rP30 is useful as an antigen for serodiagnosis of canine ehrlichiosis.

For serodiagnosis of canine ehrlichiosis, IFA is widely used. However, a fluorescence microscope and trained personnel are required for this test. Furthermore, cell culture of *E. canis* may produce batch-to-batch variation. A consistent and simple assay that can detect specific antibodies without expensive equipment would be an invaluable aid in serodiagnosis. In the dot immunoblot assay, antibody-positive serum can be distin-

guished from antibody-negative serum by the naked eye, and if proper color standards are provided, anyone can easily make the final evaluation. The greatest obstacle for the development of this assay is the production of diagnostic antigens sufficient in purity and amount. If recombinant antigens are available, the antigen preparation would be simpler, more consistent, and economical than purified organism antigen preparation. Previously, a dot blot enzyme-linked immunoassay for detecting antibodies to *E. canis* has been reported (4). However, the crude antigens, freed from host cells by freezing-thawing, were used in that study. Neither recombinant antigens nor the purified antigens (such as organisms purified by Sephacryl S-1000 column chromatography) were used. Additionally, that report contains only one page of description without any data. Therefore, we think our dot immunoblot assay using the recombinant 30-kDa antigen of *E. canis* would greatly enhance serodiagnosis of canine ehrlichiosis.

Recognition of the lowest positive IFA titer (1:20) plasma by a dot immunoblot assay with 1  $\mu$ g or less of protein of the whole organism or the recombinant antigen per dot shows that this assay is as sensitive as IFA. Although the specificity of the test, except for cross-reactivity with *E. chaffeensis*, was not analyzed in this study, as with any other serologic test, dot immunoblot assaying probably cannot distinguish among antigenically cross-reactive members of the tribe *Ehrlichieae*. However, the use of recombinant *E. canis* antigen gave greater sensitivity than the use of recombinant *E. chaffeensis* antigen for serodiagnosis of canine ehrlichiosis. Western blot analysis revealed that 8 of 22 IFA-positive plasma samples slightly cross-reacted with recombinant 28-kDa protein of *E. chaffeensis*. This weak cross-reactivity is not a potential problem for clinics, since treatment is the same for all of the ehrlichial agents.

In dot immunoblot assays of 29 IFA-positive plasma sam-

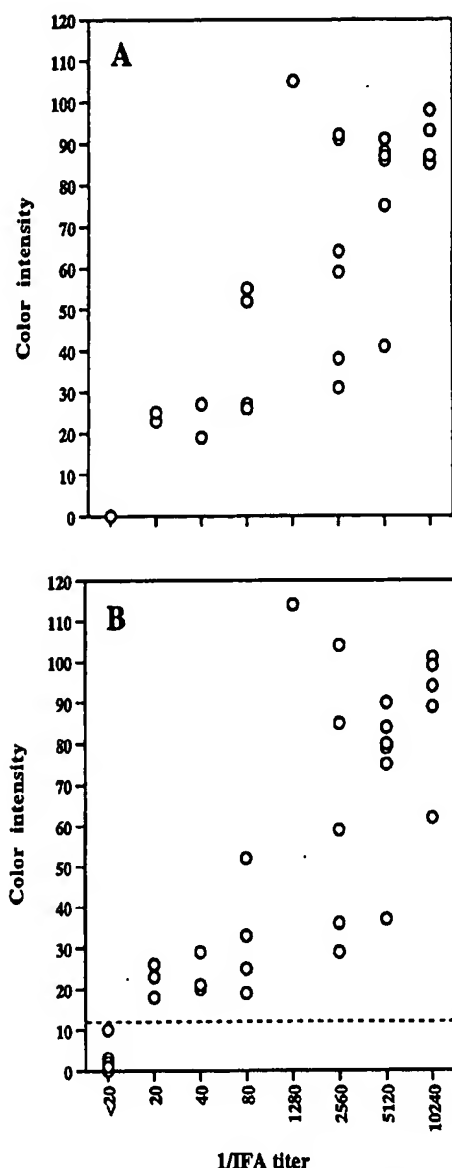


FIG. 9. Correlation between IFA titer (reciprocal dilutions) and color intensity of the dot immunoassay with purified *E. canis* antigen (A) and partially purified rP30 antigen (B). The color intensities of all dots in Fig. 8 were determined and plotted. Each circle represents one plasma specimen ( $n = 42$ ). The correlation coefficients were 0.71 ( $P < 0.001$ ) for graph A and 0.68 ( $P < 0.001$ ) for graph B. The dashed line in graph B represents the cutoff value, which was determined from the highest color intensity in the immunoreaction with 13 negative plasma samples.

ples, 5 had color intensities of the purified organism antigen greater or lesser than those of the recombinant antigens. Additional major immunodominant proteins of *Ehrlichia* spp. are heat shock proteins (HSPs) (29, 33). Consequently, when anti-HSP antibody or antibody against protein antigen other than P30 is present in the plasma, whole organism antigens would give an immunoreaction stronger than that of the recombinant protein. On the contrary, when anti-P30 antibody is dominant in the plasma, the reaction with the recombinant protein would be stronger than that with the whole organism antigen. More

importantly, the recombinant antigen-dot blot assay could clearly detect all of the 29 IFA-positive plasma samples. Furthermore, between native and recombinant antigens, no significant difference was observed in the correlation coefficient between IFA titers and the blot color intensity. Therefore, the rP30 antigen-immunodot blot assay offers advantages over the other serodiagnostic tests in general availability, ease of handling, and accuracy in the serodiagnosis of *E. canis* infection. Additionally, although it was not described in this paper, this *E. canis* recombinant antigen can be applied to enzyme-linked immunosorbent plate assays or other serodiagnostic assays as well.

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